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Suppression of Alveolar Macrophage Apoptosis Prolongs Survival of Rats and Mice with Pneumocystis Pneumonia

Mark E. Lasbury,* Pamela J. Durant,* Chad A. Ray,* Dennis Tschang,* Reto Schwendener,† and Chao-Hung Lee*

The number of alveolar macrophages is decreased in patients or animals with Pneumocystis pneumonia (Pcp). This loss of alveolar macrophages is in part due to apoptosis caused by Pneumocystis infection. The mechanism of apoptosis induction is unknown. Cell-free bronchoalveolar lavage fluids from Pneumocystis-infected rats or mice have the ability to induce apoptosis in normal alveolar macrophages. To characterize the mechanisms by which apoptosis proceeds in alveolar macrophages during Pcp, specific caspase inhibitors are tested for their ability to suppress the apoptosis. In vitro induction of apoptosis can be inhibited by the caspase-9 inhibitor (Z-LEHD-FMK) but not by the inhibitor to caspase-8 or -10. The caspase-9 inhibitor can also inhibit apoptosis of alveolar macrophages in vivo when it is intranasallyinstilled into dexamethasone-immunosuppressed, Pneumocystis-infected rats or L3T4 cell-depleted, Pneumocystis-infected mice. The number of alveolar macrophages rebounds in caspase-9 inhibitor-treated Pcp animals. Phagocytic activity of alveolar macrophages in treated animals is also recovered, and organism burden in these animals is reduced. Administration of caspase-9 inhibitor also clears the exudate that normally fills the alveoli during Pcp and decreases lung inflammation. Furthermore, caspase-9-treated Pcp animals survive for the entire 70-day period of the study, whereas nontreated Pcp animals die 40–60 days after initiation of infection. Depletion of recovered alveolar macrophages by intranasal administration of cladronate-containing liposomes in caspase-9 inhibitor-treated animals abrogates the effects of the inhibitor. Together, these results indicate that immunomodulation of the host response may be an alternative to current treatments for Pcp. The Journal of Immunology, 2006, 176: 6443–6453.

The innate immune response to Pneumocystis plays an important role in the clearance of the organism from the lung. This immune response is mediated in part by alveolar macrophages. CD4+ lymphocytes are crucial in immune response to Pneumocystis because immunosuppression leading to the loss of these cells predispose patients or animals to Pneumocystis pneumonia (Pcp) (1–4). Both Th2 and Th1 responses are activated, with liberation of IL-4 and IFN-γ (5). IFN-γ mediates Pneumocystis clearance through augmentation of alveolar macrophage function (6–8), regulates the inflammatory response during Pcp (9), and activates alveolar macrophages, while IL-4 regulates the inflammatory response. Although CD8+ lymphocytes by themselves are not sufficient to provide resistance to the infection, they do dampen the severity of the disease early in infection (10). Paradoxically, these same CD8+ cells may participate in lung damage late in disease (11, 12).

B lymphocytes also participate in the immune response because >80% of people have serum Abs to Pneumocystis by 4 years of age (13). B cell-deficient mice are susceptible to the infection (14), and administration of Pneumocystis-specific IgM Abs are partially protective in mouse models of Pcp (15–18). B cells are also important for the costimulation of T cells in response to the organism (19). T cells then activate alveolar macrophages (20, 21), which are the major type of cells responsible for the clearance of the organism from the lung.

Alveolar macrophages clear Pneumocystis through liberation of cytokines, chemokines, and growth factors (22–27); phagocytosis of organisms (28–30); and production of reactive oxygen species (31). The importance of the alveolar macrophage in clearance of Pneumocystis organisms from the lung is ascertained by the impaired response of rats when they are depleted of macrophages (29). In immunocompromised and Pneumocystis-infected hosts, the alveolar macrophages are defective, with low levels of phagocytosis (23, 32–34) and reduced NO production (34, 35). These defects are compounded by a reduction in alveolar macrophage numbers during Pcp (36–41). This decrease in alveolar macrophage number is, in part, due to apoptosis induced by Pneumocystis infection (M. E. Lasbury, S. Merali, P. J. Durant, D. Tschang, C. A. Ray, and C. H. Lee, submitted for publication). The mechanism through which Pneumocystis organisms induce apoptosis in alveolar macrophages has yet to be determined. The extrinsic pathway of apoptosis is stimulated by molecules outside the cell, typically involving the binding of extracellular factors to death domain-containing receptors on the target cell surface. The signal is transduced with activation of caspases-8 and -10. In contrast, the intrinsic pathway is stimulated within the cell, with activation of caspase-9. The activity of caspase-8 and -10 can also lead to activation of caspase-9 through cleavage of the proapoptotic Bid protein.

This study was designed to define the pathway of apoptosis activated in alveolar macrophages during Pcp, through inhibition
of activated caspases from both the extrinsic and intrinsic pathways. Experiments were also performed to determine whether suppression of apoptosis of alveolar macrophages during Pcp could be accomplished by inhibiting the activation of the initiator caspases.

Results of the study revealed that suppression of caspase-9 activity resulted in a rebound in alveolar macrophage number, recovery of phagocytic activity of alveolar macrophages, reduced organism burden and lung inflammation, and prolonged survival of infected animals.

Materials and Methods

Reagents

Caspase inhibitors were purchased from R&D Systems. These inhibitors are small synthetic peptides specific for caspase-8 (Z-IE TD-FMK), caspase-9 (Z-LEHD-FMK), and caspase-10 (Z-AYED-FMK). The Z (benzoylcarbonyl group) moiety at the nitrogen terminus aids in the passage of the molecule through cell membranes, while the FMK (fluoromethylketone group) at the C terminus makes the inhibition of the caspase enzyme irreversible. These modifications allow both in vitro and in vivo uses of the inhibitors. Anti-rat CD4-PE and CD3-FITC Ab were purchased from BD Biosciences Pharmingen. All other reagents were purchased from Sigma-Aldrich unless stated otherwise.

Rodent models of Pcp

Female Sprague Dawley rats (Harlan), 120–140 g, were used. They were divided into three groups: Normal, immunocompetent and uninfected; Dex, immunosuppressed with 1.8 mg/ml dexamethasone in drinking water; and Dex-Pc, immunosuppressed with dexamethasone and transtracheally inoculated with Pneumocystis. Female BALB/c mice (Harlan), 18–20 g, were also used. Immunosuppression of BALB/c mice was achieved by depletion of L3T4 lymphocytes using a mAb (Ab OK 1.5) as described previously (7, 42). Pneumocystis-infect ed L3T4-depleted mice are referred to as L3T4-depleted-Pc mice. Preparation of inoculum organisms (Pneumocystis carinii for rats and Pneumocystis muralis for mice) and transtracheal inoculation were performed as described previously (33, 41, 43). Pneumocystis organisms from immunosuppressed and infected or just immunosuppressed animals were identified and enumerated by scoring numbers of organisms on histochromatically stained impression smears from two separate lobes of lung tissue. The smears were stained with Wright-Giemsa stain for both trophozoite and cyst forms and with Grocott methenamine-silver nitrate stain for cyst forms (41). The slides were examined as unknowns by two experienced pathologists. Organisms were counted on 100 microscopic fields for each slide from an individual animal, and numbers were expressed as organisms per 1000 microscopic field. Infected animals that showed any bacteria were excluded; immunosuppressed control animals that had bacterial or Pneumocystis organisms identified on impression smears were also excluded.

Experimental infected animals that displayed four of the following criteria were euthanized: (a) death regardless of the experimental protocol; weight loss below 65% of arrival weight, ability to palpate bony structures such as the spine, dark appearance of the eyes, dried blood or fluid around or from the nares, inactivity, poor grooming, and hunched posture. In addition, animals were immediately euthanized when they showed signs of respiratory distress such as labored breathing. In our experience, respiratory distress is usually a later symptom seen in rats and mice with Pcp.

Animal studies were approved by the Indiana University Animal Care and Use Committee. Experiments were conducted under the supervision of veterinarians. Animals were housed in the Indiana University Laboratory Animal Resource Center, an American Association of Laboratory Animal Sciences approved facility.

Caspase inhibitor instillation in rats and mice

Some Dex-Pc rats were treated every 14 days until the study concluded by intranasal instillation with 80 μg of a specific caspase inhibitor or 80 μg each of all three inhibitors mentioned above in 100 μl of saline with 1% DMSO. The treatments began either on the day that organisms were transtracheally inoculated or 21 days later. For treatments on the same day that organisms were inoculated, caspase inhibitors were included in the inoculum. Subsequent treatments were delivered intranasally. For mice, 8 μg of inhibitors for caspase-8, -9, or -10 in 50 μl of saline/1% DMSO were delivered also either on the day of organism instillation or on day 21 of infection and then every 14 days until the end of the study. Dosage in rats was figured as eight times the dosage used in vitro (44, 45), because this study was the first time that these caspase inhibitors were used in vivo.

Mouse dosage was one-tenth of rat dose due to smaller mass. Data presented are for individual animals in a single study in which 10–20 animals were used in each condition. The data are representative of three separate studies.

Isolation of bronchoalveolar lavage (BAL) fluids and alveolar macrophages from animals

BAL fluids and alveolar macrophages were isolated from Normal, Dex, and Dex-Pc rats and Normal, L3T4-depleted, and L3T4-depleted-Pc mice as described previously (33, 41). Animals were anesthetized with an i.m. injection of ketamine mixture (ketamine hydrochloride 80 mg/ml, acepromazine 1.76 mg/ml, and atropine 0.38 μg/ml). The thoracic cavity and trachea of anesthetized rats were exposed by dissection and cutting away of the ribs. Pyrogen-free, 0.9% saline (pH 7.4) (warmed to 37°C) was injected into the lungs (5 ml for rats and 1 ml for mice) with a 10-ml syringe via an angiocath (BD Biosciences) inserted into the trachea and recovered to a sterile tube. Lavage was continued until a total of 100 ml of lavage fluid was recovered from each rat or 10 ml from each mouse. The lavage fluid was centrifuged at 300 × g for 5 min at 25°C, and the pelleted cells were suspended to 2 × 10⁷/ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 1 μM pyruvate, 1% nonessential amino acids, 14 mM glucose, 17.9 mM NaHCO₃, 10 mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were incubated on tissue culture plastic for 1 h at 37°C/5% CO₂ to allow attachment of macrophages. Nonadherent cells were removed by washing with normal saline. Isolated alveolar macrophages were identified by morphology and by their reaction with the anti-AMA Ab, which is specific for alveolar macrophages and counted on hemocytometer as described previously (20). All isolations were >95% macrophages. If necessary, purified alveolar macrophages were lifted from the tissue culture plastic by incubating with 5 mM EDTA in saline for 15 min at 37°C and gentle scraping (46). The detached cells were washed in saline, resuspended in complete medium, counted by hemocytometer, and diluted to the appropriate concentration for each experiment.

For experiments in which minimal dilution of alveolar fluids was required, the first aliquot of lavage fluid (5 ml for rats and 1 ml for mice) was kept separate. Cells and organisms were removed from the lavage fluids by centrifugation at 300 × g for 10 min, and these cells were combined with those recovered from the subsequent lavages. An aliquot of the BAL fluid supernatants was stained with Giemsa stain and assessed by hemocytometer and diluted to the appropriate concentration for each experiment.

For isolation of Pneumocystis organisms, the BAL fluid supernatants were injected into the lungs (5 ml for rats and 1 ml for mice) with a 10-ml syringe and recovered to a sterile tube. Lavage was continued until a total of 100 ml of lavage fluid was recovered from each rat or 10 ml from each mouse. The lavage fluid was centrifuged at 300 × g for 5 min at 25°C, and the pelleted cells were suspended to 2 × 10⁷/ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 1 μM pyruvate, 1% nonessential amino acids, 14 mM glucose, 17.9 mM NaHCO₃, 10 mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were incubated on tissue culture plastic for 1 h at 37°C/5% CO₂ to allow attachment of macrophages. Nonadherent cells were removed by washing with normal saline. Isolated alveolar macrophages were identified by morphology and by their reaction with the anti-AMA Ab, which is specific for alveolar macrophages and counted on hemocytometer as described previously (20). All isolations were >95% macrophages. If necessary, purified alveolar macrophages were lifted from the tissue culture plastic by incubating with 5 mM EDTA in saline for 15 min at 37°C and gentle scraping (46). The detached cells were washed in saline, resuspended in complete medium, counted by hemocytometer, and diluted to the appropriate concentration for each experiment.

Pneumocystis viability assay

For isolation of Pneumocystis organisms, the BAL fluid supernatants were injected into the lungs (5 ml for rats and 1 ml for mice) with a 10-ml syringe and recovered to a sterile tube. Lavage was continued until a total of 100 ml of lavage fluid was recovered from each rat or 10 ml from each mouse. The lavage fluid was centrifuged at 300 × g for 5 min at 25°C, and the pelleted cells were suspended to 2 × 10⁷/ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 1 μM pyruvate, 1% nonessential amino acids, 14 mM glucose, 17.9 mM NaHCO₃, 10 mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were incubated on tissue culture plastic for 1 h at 37°C/5% CO₂ to allow attachment of macrophages. Nonadherent cells were removed by washing with normal saline. Isolated alveolar macrophages were identified by morphology and by their reaction with the anti-AMA Ab, which is specific for alveolar macrophages and counted on hemocytometer as described previously (20). All isolations were >95% macrophages. If necessary, purified alveolar macrophages were lifted from the tissue culture plastic by incubating with 5 mM EDTA in saline for 15 min at 37°C and gentle scraping (46). The detached cells were washed in saline, resuspended in complete medium, counted by hemocytometer, and diluted to the appropriate concentration for each experiment.

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TUNEL assay

DNA fragmentation in alveolar macrophages was examined as a measure of apoptosis by the TUNEL assay. Isolated alveolar macrophages were fixed in 4% paraformaldehyde for 25 min, washed twice in PBS for 5 min each, and permeabilized in PBS containing 0.2% Triton X-100 for 5 min. After rinsing again in PBS, the slides were covered with equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM cobalt chloride) for 5 min and then incubated with the biotinylated nucleotide mix (1:100) and terminal deoxynucleotidyl transferase (1:100) from the DeadEnd Colorimetric TUNEL Assay Kit (Promega) for 60 min at 37°C. The slides were washed in 2× SSC for 15 min, rinsed in PBS, and then treated with 0.3% hydrogen peroxide for 5 min to inactivate the endogenous peroxidase activity. The hydrogen peroxide was washed off with PBS, and the slides were incubated in 100 μl of streptavidin-HRP (1:500) for 30 min. After washing in PBS to remove the excess streptavidin-HRP, the slides were incubated with diaminobenzidine substrate (1:50), diaminobenzidine chromagen (1:20), and 5 μl of hydrogen peroxide until the stain was visible. At least 300 alveolar macrophages were counted from each slide, and average percentage of apoptotic macrophages ± SD was obtained for at least five animals or 10 independent alveolar macrophage aliquots from each condition.

Immunophenotyping of peripheral blood and BAL cells

Two milliliters of whole blood was collected in 1.8 mg/ml EDTA-containing tubes (Fisher Scientific) from animals during cardiac exsanguination. Blood was stored at 4°C until analyzed. BAL fluids, including the BAL cells, were isolated as described above, and 2 ml of aliquots were assessed immediately. Anti-rat CD4-PE and CD3-FITC Abs (BD Biosciences Pharmingen) were added (5 μl at 20 μg/ml) to 100 μl of whole blood for 15 min at 25°C. Erythrocytes were lysed with 600 μl Immunoprep Reagent A (Beckman Coulter), whereas leukocytes were stabilized with 265 μl of Immunoprep Reagent B (Beckman Coulter). Cells were fixed with Immunoprep Reagent C (Beckman Coulter) and 400 μl of 1% paraformaldehyde. To normalize the volumes of sample counted, 100 μl (1058 beads/μl) of Coulter beads were added to each sample, and the samples were analyzed on a FC500 flow cytometer (Beckman Coulter). Markers were set to exclude >95% of cells stained with appropriate isotype control Abs. Results indicate the number of CD4+ cells per milliliter.

Alveolar macrophage depletion

Depletion of alveolar macrophages in rats was achieved by intranasal instillation of 2 mg of clodronate-containing liposomes (20 mg/ml clodronic acid disodium salt) as described previously (50). Rats of the same condition were instilled with empty liposomes as controls for liposome effects. Counting of alveolar macrophages in BAL fluids from test animals indicated that this method depleted >94% of the alveolar macrophages for a 5-day period, so instillations were performed every 5 days until the animals were sacrificed.

Statistics

Comparisons were made between the mean values of the treatment and control groups or between two treatment groups by paired or unpaired Student’s t test where appropriate, using a two-tail distribution. Comparisons between three or more treatment groups were made by ANOVA. Planned comparison tests as follow-up to ANOVA included both Bonferroni’s t test and multiple paired t tests for groups identified as statistically different. A p value <0.05 was considered significant.

Results

Alveolar macrophage apoptosis induced by BAL fluids from infected animals is suppressed by caspase-9 inhibitor

To determine whether apoptosis of alveolar macrophages during Pcp is mediated by the extrinsic or intrinsic apoptosis pathway, inhibitors to initiator caspases including caspases 8, 9, and 10 were tested for their ability to block the apoptosis. Normal alveolar macrophages were incubated in 500 μl of Dex-Pc BAL fluids for 48 h, with or without 20 μM (10 μg/ml) caspase inhibitors. Dosages of caspase inhibitors were equal to those used in published reports (44, 45). As shown in Fig. 1, 17.2 ± 2.0% of cells incubated with Dex-Pc BAL fluids became apoptotic with DNA fragmentation detected by the TUNEL assay. An aliquot of cells incubated with Dex-Pc BAL fluids and 20 μM of a glucocorticoid receptor antagonist (RU486) also had a high rate of apoptosis, indicating that the dexamethasone which might be present in the BAL fluids was
Pneumocystis carinii. To determine whether the caspase inhibitors are effective in macrophage apoptosis in rats and mice with Pcp, in vivo instillation of caspase-9 inhibitor suppresses alveolar macrophage apoptosis rate to 7.7 ± 1.1% (p < 0.05) when a caspase-9 inhibitor was present in the L3T4-depleted-Pc BAL fluids (Fig. 1). Inclusion of caspase-8 or caspase-10 inhibitor did not suppress the apoptosis induced by the BAL fluids (p > 0.05). These data suggest that inhibition of caspase-9 activation could suppress the apoptosis of alveolar macrophages and that the intrinsic pathway of apoptosis is of primary importance in response to Dex-Pc BAL fluids.

In vivo instillation of caspase-9 inhibitor suppresses alveolar macrophage apoptosis in rats and mice with Pcp

To determine whether the caspase inhibitors are effective in vivo, immunosuppressed rats were given P. carinii inocula that also contained caspase inhibitor or an equal volume of carrier. The caspase inhibitor or carrier alone was then intranasally instilled into the rats every 14 days. Animals were sacrificed when moribund or on day 70 of the study, and alveolar macrophages from these rats were assessed for apoptosis by the TUNEL DNA fragmentation assay. Rats treated with carrier alone manifested severe infections with a significant rate of alveolar macrophage apoptosis. Alveolar macrophages from rats that received the caspase-8 or -10 inhibitor showed no reduction in the apoptosis rates (Fig. 2). In contrast, instillation of caspase-9 inhibitor decreased the number of apoptotic alveolar macrophages by 62.6% (p < 0.0001 vs control). When all three caspase inhibitors were used, the apoptosis rate was reduced by 66.5%, similar to those treated with the caspase-9 inhibitor alone (Fig. 2). These results, again, suggest that caspase-8 and -10 do not play a major role in the stimulation of apoptosis of alveolar macrophages in Pneumocystis infection.

In the L3T4-depleted mouse model of Pcp, alveolar macrophage apoptosis showed the same profile as in rats. Caspase-9 inhibitor reduced apoptotic cells by 67.3% as compared with carrier controls (Fig. 2). As seen in rats, caspase-8 and -10 inhibitors had no suppressive effect on alveolar macrophage apoptosis, and the apoptosis rates of Pcp mice treated with these two inhibitors stayed high (Fig. 2).

The number of alveolar macrophages in these animals correlated with the TUNEL data. Dex-Pc rats treated with the caspase-8 or -10 inhibitor showed no improvement in the severity of infection, and the decreases in alveolar macrophage number were as large as the untreated controls (Fig. 3). In contrast, rats treated with the caspase-9 inhibitor showed smaller losses of alveolar macrophages (p < 0.05 vs untreated controls, caspase-8 or -10 inhibitor-treated animals; Fig. 3).

Mice with Pcp also showed higher alveolar macrophage numbers when caspase-9 inhibitor was instilled at the same time as organisms, with the alveolar macrophage number rebounded 271% as compared with controls (p < 0.05 vs untreated controls, caspase-8 or -10 inhibitor-treated animals; Fig. 3). Similar to that observed in rats, the caspase-8 or -10 inhibitor had no effect.

Suppression of alveolar macrophage apoptosis during Pcp reduces organism burden

The proliferation of Pneumocystis in the lung was inhibited when alveolar macrophage apoptosis was suppressed. In rats treated with caspase-9 inhibitor or all three inhibitors, trophozoite burdens were 63–65% lower than those of untreated moribund animals or those that were treated for the same period of time with the caspase-8 or -10 inhibitor (Fig. 4A). Similarly, cyst burdens were reduced 70% in caspase-9 inhibitor-treated animals, as compared with those from untreated or caspase-8 or -10 inhibitor-treated rats (Fig. 4A). This result indicates that inhibition of macrophage apoptosis had a suppressive effect on the replication of the organism.

Trophozoite and cyst burdens were also reduced in the mouse model of Pcp when caspase-9 was inhibited. Trophozoites were reduced by 71% in Pcp mice treated with the caspase-9 inhibitor as

FIGURE 3. Alveolar macrophage numbers in BAL fluids from caspase inhibitor-treated Pcp rats and mice. Rats and mice with Pcp were treated with caspase inhibitors (80 μg for rats and 8 μg for mice) every 14 days after instillation of organisms. Alveolar macrophages were counted in BAL fluids when the animals met the criteria for sacrifice or when they were sacrificed at the end of the study period. Dex rats and L3T4 mice were the immunosuppressed controls, whereas Dex-Pc rats and L3T4-Pc mice were infected control animals. Immunosuppressed represents Dex rats or L3T4-depleted mice, and infected represents Dex-Pc rats or L3T4-Pc mice. Values are averages ± SD for 15–19 animals of each condition. *, p < 0.05 vs untreated control of the same species.
untreated rats (Fig. 5), as well as in those of mice (Fig. 5) and showed decreased viability (46.1 ± 2.6% for *P. carinii* and 34.9 ± 5.6% for *P. murina*) after 48 h. Caspase-8 inhibitor treatment reduced the viability of *P. carinii* and *P. murina* by only 1.4 and 2.2%, respectively. Viability in caspase-9 inhibitor (92.5 ± 3.6% for *P. carinii* and 90.6 ± 7.2% for *P. murina*) and caspase-10 inhibitor (91.7 ± 4.1% for *P. carinii* and 89.2 ± 10.2% for *P. murina*) treated cultures were similar to carrier only treated control cultures (*p* > 0.05).

**Suppression of alveolar macrophage apoptosis during Pcp restored phagocytic ability**

To investigate the reason for decreased organism burden when Pcp-mediated alveolar macrophage apoptosis was suppressed, phagocytic ability of these cells after caspase-9 inhibition was assessed. As previously described, alveolar macrophages from Dex-Pc rats are similarly defective in phagocytosis of latex beads and radiolabeled *Pneumocystis* organisms (33); therefore, latex beads were used to study phagocytic ability in these cells. In this study, alveolar macrophages from Dex rats phagocytosed 21.6 ± 4.2 latex beads, whereas those from Dex-Pc rats phagocytosed an average of only 1.46 ± 0.2 latex beads per cell after 1 h of incubation (Fig. 6A); both results were in agreement with previous results (33). Alveolar macrophages from rats treated with caspase-9 inhibitor every 14 days beginning at the time when organisms were instilled internalized an average of 14.9 ± 3.1 latex beads (Fig. 6A; *p* < 0.0001 vs untreated cells). As shown in Fig. 6B, alveolar macrophages from Dex-Pc rats had many latex beads bound to the cell, but few were internalized. Alveolar macrophages from caspase-9 inhibitor-treated Dex-Pc rats had many bound latex beads, as well as many internalized beads (Fig. 6C). Confocal microscopy confirmed that the beads were indeed internalized (data not shown). This result suggests that the reduced organism burden in Dex-Pc rats treated with caspase-9 inhibitor is due, in part, to increased phagocytic ability of the alveolar macrophages.

**Inhibition of caspase-9 increases survival time of rats and mice with Pcp**

The survival rates of Pcp animals treated with caspase inhibitors were also determined. Results shown in Fig. 7A indicate that rats treated with carrier alone or caspase-8 or -10 inhibitor became severely ill and had to be euthanized beginning day 34 of the studies, according to the criteria described above. In contrast, all rats that received caspase-9 inhibitor or all three inhibitors survived to the conclusion of the study and were sacrificed at day 70 (Fig. 7A).

L3T4-depleted-Pc mice treated with caspase inhibitors showed similar survival curves. Untreated animals required euthanasia beginning on day 44 and were all sacrificed by day 64 (Fig. 7B). However, all Pcp mice that were treated with caspase-9 inhibitor survived until the study was terminated at day 72 (Fig. 7B). The survival results in both Pcp rats and mice treated with caspase inhibitors indicate that suppression of alveolar macrophage apoptosis resulted in delayed progression of the infection, although the caspase-9 inhibitor treatment did not completely resolve the infection within the study period.

Compared with the untreated control Pcp mice (Fig. 4B), the inhibitors to caspase-8 and caspase-10 did not reduce the trophozoite burden in L3T4-depleted-Pc mice (Fig. 4B). Similarly, the cyst burden was reduced in caspase-9 inhibitor-treated mice (decreased 70%) as compared with untreated controls, but caspase-8 and caspase-10 inhibitors did not decrease the number of cysts in the lungs of the infected animals (Fig. 4B).

As seen in Fig. 5, many cysts were apparent in lung smears of untreated rats (Fig. 5A) and mice (Fig. 5B), as well as in those of animals treated with the inhibitors to caspases-8 and -10. But, rats treated with caspase-9 inhibitor or all three inhibitors had many fewer cysts (Fig. 5A), as did mice treated with the caspase-9 inhibitor (Fig. 5B).

It was possible that the reduced organism burden in caspase inhibitor-treated animals was due to a direct toxicity of the caspase-9 inhibitor to the *Pneumocystis* organism. To investigate this possibility, we incubated *P. carinii* and *P. murina* mixed populations isolated from infected animals with the caspase inhibitors. Calcein M and ethidium bromide staining of the organisms after 48-h incubation with inhibitor showed no increase in cell death as compared with those organisms incubated with carrier alone. In this experiment, 91.2 ± 4.9% of *P. carinii* and 88.2 ± 9.1% of *P. murina* organisms were viable after incubation with carrier, whereas organisms treated with trimethoprim/sulfamethoxazole showed decreased viability (46.1 ± 2.6% for *P. carinii* and 34.9 ± 5.6% for *P. murina*) after 48 h. Caspase-8 inhibitor treatment reduced the viability of *P. carinii* and *P. murina* by only 1.4 and 2.2%, respectively.
Treatment of established Pcp in rats and mice with caspase-9 inhibitor

The above results show that inhibition of caspase-9 in rats and mice with Pcp increases survival and reduces organism burden and inflammation when the inhibitor treatment was begun at the same time as the infection was initiated. To determine whether inhibiting caspase-9 could improve the condition of animals with established Pcp, caspase-9 inhibitor treatment was started 21 days after initiation of the infection. At this stage of the infection, there was a measurable organism burden and reduced alveolar macrophage numbers in rats (41), but a transient increase in alveolar macrophages with a measurable organism burden in mice (M. E. Lasbury, S. Merali, P. J. Durant, D. Tschang, C. A. Ray, and C. H. Lee, submitted for publication). Some animals received one treatment of caspase-9 inhibitor, and others received a second treatment 14 days later. Animals were sacrificed 7 days after the final treatment. In rats treated in this way, the alveolar macrophage number increased by 75% after one treatment and 95% after two treatments as compared with those of untreated rats (Table I). This increase was accompanied by a decrease in the apoptosis rate of these cells for animals treated either once or twice ($p < 0.05$ for both vs untreated). Similar to rats treated from the beginning of the infection, the organism burdens in these rats were reduced. Trophozoite burden was reduced by 74% in those treated once and 86% in those treated twice ($p < 0.05$ vs untreated), and cyst burden was reduced by 78 and 93% for once and twice treated, respectively (Table I).

In Pcp mice, the rates of alveolar macrophage apoptosis were decreased by 33% for one treatment and 65% for two treatments. The trophozoite organism burdens were reduced by 65% for one treatment and 81% for two treatments (Table II). In each case, the change was significant as compared with untreated mice ($p < 0.05$), and the change was greater in animals treated twice ($p < 0.05$ compared with once treated).

These results suggest that inhibition of caspase-9 not only prevents progression of the infection, but also cures established infections.

Alveolar exudate and inflammation is reduced with caspase-9 inhibition in Pcp

To determine whether the treatment with caspase inhibitors also brought changes in the lung histology, we examined sections of lung tissue from infected and caspase inhibitor-treated animals. Rats treated with caspase-8 or -10 inhibitor showed the same lung pathology as that seen in untreated rats (Fig. 8A). The alveoli of untreated rats were filled with exudate, and the lung had thickened alveolar septa and few alveolar macrophages. Pcp rats treated with caspase-8 or caspase-10 inhibitor had the same levels of exudate and inflammation as the untreated rats. In contrast, rats treated with
caspase-9 inhibitor or all three inhibitors showed clear alveoli and the presence of alveolar macrophages in the alveoli (Fig. 8A).

In the L3T4-depleted mouse model, *Pneumocystis*-infected mice had alveolar exudate and thickened alveolar walls, with no alveolar macrophages apparent. Treatment with caspase-9 inhibitor (Fig. 8B) resulted in much less alveolar exudate and thinner alveolar walls. These results suggest that one reason for prolonged survival in caspase-9 inhibitor-treated animals is the reduced alveolar filling, thus increasing gas exchange and decreasing damage to the lung.

**Caspase-9 inhibitor does not restore CD4⁺ cell numbers in Pcp rats**

To confirm that the less severe disease in Pcp animals treated with caspase-9 inhibitor was not due to rescue of the CD4⁺ cells that were lost by dexamethasone treatment, we measured the CD4⁺ cells in the peripheral blood and BAL fluids from these animals 5 days after the final inhibitor instillation. The pan lymphocyte marker CD3 was used to enumerate lymphocytes, and the percentage and number of CD3⁺ cells that also expressed the CD4 marker were determined. In immunocompetent rats, 19.8% of lymphocytes in the blood were high expressors of both CD3 and CD4. Although 95.1% of lymphocytes in Dex-Pc rats expressed CD3, only 3.6% of these cells were also positive for CD4. This decrease in CD4⁺ cells was not changed in the Dex-Pc rats that were treated with caspase-9 inhibitor. The loss of CD4⁺ cells induced by dexamethasone was not reversed by caspase-9 inhibitor 5 days after the final caspase inhibitor instillation. Similarly, in BAL fluids from Normal rats, 88.9% of the CD3⁺ cells were also CD4⁺, whereas only 9.4% were both CD3 and CD4 positive in those from Dex-Pc rats. This percentage did not change appreciably in Dex-Pc rats treated with caspase-9 inhibitor (10.6%; *p* < 0.05 vs Dex and Dex-Pc controls).

**Macrophage depletion abrogates the prolonged survival resulting from caspase-9 inhibitor treatment in Dex-Pc rats**

To determine whether rescue of alveolar macrophages alone was the reason for increased animal survival, we depleted alveolar macrophages in untreated and caspase-9 inhibitor-treated Pcp rats. Two milligrams of clodronate-containing liposomes was instilled intranasally every 5 days into some rats that were receiving...
caspase-9 inhibitor. As a control, empty liposomes were instilled into some untreated and treated rats. Fifteen to 21 animals were assessed for each condition. Empty liposomes had a negligible effect on disease progression in rats of any condition. As shown in Fig. 9, all Dex-Pc rats became agonal starting on day 40, and all animals of this condition had to be sacrificed by day 47. When Dex-Pc rats were depleted of alveolar macrophages with clodronate-containing liposomes, they reached a terminal stage of disease beginning on day 32, 8 days sooner than those Dex-Pc rats that were not macrophage depleted. All of these animals met the criteria for euthanasia described above and were sacrificed by day 43 (Fig. 9). In caspase-9 inhibitor-treated Dex-Pc rats, all animals survived until the study was terminated, as did animals treated with empty liposomes. Macrophage depletion caused the inhibitor-treated animals to expire at rates and times similar to those of untreated Dex-Pc rats (Fig. 9). This result clearly shows that the suppression of the alveolar macrophage apoptosis is the reason why the caspase-9 inhibitor-treated animals survived longer.

Discussion

Results of this study indicate that suppression of alveolar macrophage apoptosis delays or halts Pcp progression in rats and mice. Investigation was focused on the initiator caspases including caspases 8, 9, and 10, and inhibitors to these caspases were examined for their ability to inhibit apoptosis of alveolar macrophages. Normal alveolar macrophages challenged with apoptosis-inducing BAL fluids from infected rats and mice were able to resist the apoptotic stimulus when an inhibitor to caspase-9 was included in the apoptotic stimulus when an inhibitor to caspase-9 was included in the incubations. Interestingly, this inhibitor was also effective in the treatment of Pcp in immunosuppressed rats and mice. The use of all three caspase inhibitors in Pneumocystis-infected rats did not have an additive or synergistic effect on suppression of the infection (Figs. 2–5, 7, 8), suggesting that caspase-9 is the major enzyme involved in the apoptosis. The observation that only caspase-9 inhibitor had a suppressive effect on disease progression confirms the relative importance of the intrinsic apoptosis pathway in these animals. The lack of suppressive effects of inhibitor to caspase-8 or -10 in the progression of Pcp suggests that the extrinsic apoptosis pathway does not play a major role in the apoptosis of alveolar macrophages during Pcp.

Both dexamethasone-suppressed rats and L3T4-depleted mice were used in this study. Dexamethasone mediates a loss of CD4+ thymocytes by inducing their programmed cell death (51, 52). Therefore, we determined whether the caspase-9 inhibitor also suppressed the apoptosis of CD4+ thymocytes and found that CD4+ cell numbers in the peripheral blood or BAL fluids of animals treated with caspase-9 inhibitor did not increase. This result indicates that the caspase-9 inhibitor rescues alveolar macrophages, not CD4+ cells, and suggests that CD4+ cell apoptosis is mediated by a different mechanism. Previous results showed that mice respond to Pcp with an initial increase followed by a crash in alveolar macrophage number (M. E. Lasbury, P. J. Durant, D. Tschang, C. A. Ray, and C. H. Lee, submitted for publication), whereas rats showed a drop in number right after the initiation of infection (41). However, both rats and mice showed similar levels and mechanisms of apoptosis of macrophages in infection and improvement in phagocytic activity of alveolar macrophages after caspase-9 inhibition. The major difference was that inhibition of caspase-9 resulted in a rebound in alveolar macrophage number in Pcp rats at any time point during the infection but not in Pcp mice during the early stage of infection. This result suggests that mechanisms other than apoptosis are responsible for mediating alveolar macrophage number in mice at this point in the infection.

The caspase inhibitors used in this study were synthetic peptides with a benzoyloxycarbonyl group added to the N-terminal end to promote diffusion into the cell and a fluoromethylketone moiety added to the C-terminal end to render their binding to caspases irreversible. Despite these modifications, the inhibitors were susceptible to degradation by intracellular proteases. The half-life of these inhibitors in culture medium is ~24 h (G. Zhang, unpublished observations), but it is not known whether the intracellular environment protects the inhibitors or promotes their degradation. Our results indicate that the caspase-9 inhibitor at the dosage of 0.67 mg/kg instilled every 14 days was sufficient to provide a measurable decrease in Pcp progression in both rats and mice. In animals treated with caspase-9 inhibitor after the infection had been established (Tables I and II), two treatments 14 days apart was more effective in reducing organism burden and increasing alveolar macrophage number as compared with a single treatment.

Table I.  Effect of caspase-9 inhibition on established Pneumocystis infection in rats

<table>
<thead>
<tr>
<th>Condition</th>
<th>Alveolar Macrophages (×10^6)</th>
<th>TUNEL-Positive Cells (%)</th>
<th>Trophozoites/×1000 Field</th>
<th>Cysts/×1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dex-Pc + PBS</td>
<td>1.97 ± 0.2</td>
<td>39.6 ± 4.1</td>
<td>35.3 ± 2.6</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>Dex-Pc + inhibitor-9 treated once</td>
<td>3.46 ± 0.2*</td>
<td>23.1 ± 0.8*</td>
<td>9.2 ± 0.8*</td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>Dex-Pc + inhibitor-9 treated twice</td>
<td>3.86 ± 0.3*</td>
<td>14.3 ± 0.6*</td>
<td>4.9 ± 0.6*</td>
<td>0.3 ± 0.1*</td>
</tr>
</tbody>
</table>

*a Eight to 12 rats/condition.
*p < 0.05 vs PBS control.
† p < 0.05 vs once treated.

Table II.  Effect of caspase-9 inhibition on established Pneumocystis infection in mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>Alveolar Macrophages (×10^6)</th>
<th>TUNEL-Positive Cells (%)</th>
<th>Trophozoites/×1000 Field</th>
<th>Cysts/×1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3T4-Pc + PBS</td>
<td>13.4 ± 0.8</td>
<td>38.1 ± 5.1</td>
<td>14.9 ± 2.2</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>L3T4-Pc + inhibitor-9 treated once</td>
<td>14.1 ± 1.0</td>
<td>22.9 ± 4.3*</td>
<td>5.2 ± 0.3*</td>
<td>0.9 ± 0.1*</td>
</tr>
<tr>
<td>L3T4-Pc + inhibitor-9 treated twice</td>
<td>14.3 ± 0.8</td>
<td>13.4 ± 2.1*</td>
<td>2.9 ± 0.1*</td>
<td>0.2 ± 0.0*</td>
</tr>
</tbody>
</table>

*a Ten to 13 mice/condition.
*p < 0.05 vs PBS control.
† p < 0.05 vs once treated.
This result suggests that some degradation of the molecules did take place.

Further proof that suppression of alveolar macrophage apoptosis was responsible for prolonged survival and reduced disease severity was from results of the macrophage depletion experiment. Alveolar macrophage depletion with clodronate-containing liposomes eliminated the survival advantage of Pcp rats treated with caspase-9 inhibitor (Fig. 9). Clodronate and caspase-9 inhibitor-treated animals differed from caspase-9 inhibitor-treated rats, but reappeared in animals treated with the caspase-9 inhibitor. Results shown are representative of 12-15 rats from each condition. Scale bar, 100 μm.

A surprising finding in this study was that treatment of Pcp animals with the caspase-9 inhibitor also restored some phagocytic activity of alveolar macrophages (Fig. 6). The reason for this recovery in function is not apparent from these experiments. It is possible that macrophage to macrophage signaling plays a role in maintaining the function of the cells present in the lung. This signaling may be mediated by TNF-α, which activates macrophages in conjunction with IFN-γ. Macrophages also release IL-1α and IL-1β, which stimulate other macrophages to release cytokines and chemokines. In addition, macrophages produce and secrete MIP-1α, which attracts other macrophages and induces a respiratory burst. Macrophages and monocytes also liberate G-CSF, M-CSF, and GM-CSF, which promote differentiation of monocytes to macrophages. Rescue of cells from apoptosis may increase their ability to produce chemokines and cytokines to improve the functional abilities of remaining cells. In fact, GM-CSF (27) and IFN-γ (6) administration have been shown to suppress Pcp progression in rats and mice. In these ways, macrophages may modulate the number and function of other macrophages. This may be a reason why rescue of alveolar macrophages during Pcp restored phagocytic activity and reduced disease severity.

The results of this study show that an infectious disease can be controlled by immunomodulation. In this case, the organism-induced programmed cell death is suppressed by inhibiting caspase-9 activity, leading to increased alveolar macrophage number and function and prolonged survival of Pneumocystis-infected animals. This study represents the first evidence that an inhibitor of a caspase enzyme can improve the prognosis of an infectious disease in vivo. In contrast, there are examples that inhibition of apoptosis exacerbates disease, as in the case of increased bacteremia in pneumococcal pulmonary infection (53). Inhibition of caspase-9...
also has been shown to increase apoptosis of B-lineage cells in response to stress (54). These results indicate that in-depth studies of the effects of caspase inhibition are needed to discern whether unforeseen responses may accompany the inhibition of this enzyme in patients with Pcp.

Several other infectious organisms also induce apoptosis of host cells, including Mycobacterium tuberculosis (55–57), Cryptosporidium parvum (58), Shigella flexneri (59), and Helicobacter pylori (60, 61). Similar to Pneumocystis, these microorganisms also cause apoptosis through the intrinsic apoptotic pathway. The best characterized mechanism of apoptosis in infectious disease processes is in H. pylori, where apoptosis of macrophages is stimulated by reactive oxygen species resulting from the catabolism of polyamines (60). The results of this study that inhibition of apoptosis of alveolar macrophages can cure Pcp will serve as a model for investigation of similar approaches to treat other diseases.

Disclosures

The authors have no financial conflict of interest.

References

26. Krishnan, V. L., A. Meager, D. M. Mitchell, and A. J. Pinching. 1990. Alveolar macrophage and alveolar macrophage-depleted rats. Dex-Pc, rats treated with caspase-9 inhibitor every 14 days, beginning the same day that organisms were instilled. Starting 21 days after instillation of organisms, some rats were intranasally treated with clodronate-containing liposomes; Dex-Pc-lipo control, Dex-Pc rats treated with empty liposomes; Dex-Pc lipo; Dex-Pc rats treated with empty liposomes; Dex-Pc inh, Dex-Pc rats treated with caspase-9 inhibitor; Dex-Pc-inh-lipo, Dex-Pc rats treated with clodronate-containing liposomes; Dex-Pc-lipo control, Dex-Pc rats treated with empty liposomes; Dex-Pc inh, Dex-Pc rats treated with caspase-9 inhibitor; Dex-Pc-inh-lipo, Dex-Pc rats treated with clodronate-containing liposomes; Dex-Pc-lipo control, Dex-Pc rats treated with caspase-9 inhibitor and empty liposomes.